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Reduced Intracellular c-di-GMP Content Increases Expression of Quorum Sensing-Regulated Genes in *Pseudomonas aeruginosa*

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Cyclic-di-GMP (c-di-GMP) is an intracellular secondary messenger which controls the biofilm life cycle in many bacterial species. High intracellular c-di-GMP content enhances biofilm formation via the reduction of motility and production of biofilm matrix, while low c-di-GMP content in biofilm cells leads to increased motility and biofilm dispersal. While the effect of high c-di-GMP levels on bacterial lifestyles is well studied, the physiology of cells at low c-di-GMP levels remains unclear. Here, we showed that *Pseudomonas aeruginosa* cells with high and low intracellular c-di-GMP contents possessed distinct transcriptome profiles. There were 535 genes being upregulated and 432 genes downregulated in cells with low c-di-GMP, as compared to cells with high c-di-GMP. Interestingly, both *rhl* and *pqs* quorum-sensing (QS) operons were expressed at higher levels in cells with low intracellular c-di-GMP content compared with cells with higher c-di-GMP content. The induced expression of *pqs* and *rhl* QS required a functional PqsR, the transcriptional regulator of *pqs* QS. Next, we observed increased production of *pqs* and *rhl*-regulated virulence factors, such as pyocyanin and rhamnolipids, in *P. aeruginosa* cells with low c-di-GMP levels, conferring them with increased intracellular survival rates and cytotoxicity against murine macrophages. Hence, our data suggested that low intracellular c-di-GMP levels in bacteria could induce QS-regulated virulence, in particular rhamnolipids that cripple the cellular components of the innate immune system.

Keywords: *Pseudomonas aeruginosa*, cyclic-di-GMP, quorum sensing, PQS, rhamnolipids

INTRODUCTION

Pseudomonas aeruginosa can cause opportunistic infections in humans, such as cystic fibrosis lung infections, burn wounds and urinary tract infections (Bodey et al., 1983). This is attributed to its ability to form biofilms and produce an abundance of virulence factors to impair the host immune response (Bjarnsholt et al., 2009; Fazli et al., 2011).

Similar to many Gram-negative bacteria species, the biofilm and planktonic lifestyles in *P. aeruginosa* are controlled by the secondary messenger bis-(3'-5')-cyclic-dimeric-GMP (c-di-GMP) (Romling et al., 2005). C-di-GMP is synthesized by diguanylate cyclases (DGCs) and

degraded by phosphodiesterases (PDEs) (Hengge, 2009). High intracellular c-di-GMP content enhances biofilm formation, whereas low intracellular c-di-GMP content leads to biofilm dispersal and the return to planktonic phase (Hisert et al., 2005; Romling et al., 2005; Kulasakara et al., 2006; Chua et al., 2014; Yu et al., 2015). The redundancy of DGC and PDE genes in the genome confers *P. aeruginosa* the survival advantage to respond to various stresses from the environment. For instance, the *wspR* DGC is important in the sensing of reactive oxygen species (ROS) and formation of biofilms resilient to ROS stress (Chua et al., 2016a).

Another system that plays important roles in biofilm formation and virulence is quorum sensing (QS), which is the intercellular communication system positively dependent on cell density and QS autoinducer (AI) concentrations (Fuqua et al., 1994; Whitehead et al., 2001; Ng and Bassler, 2009). *P. aeruginosa* possesses four major QS systems, encoded by the *las*, *rhl*, *pqs* and *iqs* systems, with the *las* and *rhl* systems employing homoserine lactones, namely the N-(3-oxododecanoyl)-homoserine lactone (OdDHL) and N-butanoyl-L-homoserine lactone (BHL) respectively as their AIs (Gambello and Iglewski, 1991; Passador et al., 1993; Ochsner and Reiser, 1995; Pearson et al., 1995), while *pqs* and *iqs* systems using the 2-heptyl-3-hydroxy-4(1H)-quinolone (PQS) and 2-(2-hydroxyphenyl)-thiazole-4-carbaldehyde respectively (Cao et al., 2001; Diggle et al., 2007; Lee et al., 2013). The AIs will bind and activate the transcriptional regulators, resulting in the transcription of downstream QS operons. The QS systems interregulate one another, notably the *las* system and *pqs* system activate the *rhl* system (Pesci et al., 1997; McKnight et al., 2000; Farrow et al., 2008). The QS systems control the production of many virulence factors, such as pyocyanin (by the *pqs* operon) and rhamnolipids (by the *rhl* operon) (Pearson et al., 1997; Xiao et al., 2006). The rhamnolipids are biosurfactants which are highly cytotoxic to eukaryotic cells, as previously demonstrated by the induction of *rhl* operon-controlled gene expression in biofilm bacteria exposed to polymorphonuclear leukocytes (PMNs) and subsequent destruction of these important defensive immune cells (Alhede et al., 2009).

While the impact of high c-di-GMP content on biofilm formation is well understood, the consequences of low intracellular c-di-GMP content other than biofilm dispersal remain unclear. Our previous study showed that freshly dispersed cells, during the short-term liberation process, appeared to be highly virulent as compared to biofilm cells (Chua et al., 2014). It remains elusive whether reduced c-di-GMP content may have a long-term impact on bacterial physiology and virulence.

Hence, we aimed to investigate the impact of low vs. high c-di-GMP concentrations on *P. aeruginosa* virulence mechanisms. We compared the transcriptomes of *P. aeruginosa* PAO1 cells “locked” in a condition with high c-di-GMP content (by using the *wspF* mutation to induce constitutive expression of WspR) and the cells “locked” in a condition with low c-di-GMP content (by over expressing the YhJH PDE) cultivated under the similar growth conditions. As the WspF protein is the inhibitor of the WspR DGC, the *wspF* mutation will cause expression of WspR, thereby promoting the synthesis of c-di-GMP leading to high

internal levels (Hickman et al., 2005). The PAO1/*p_{lac}-yhjH* strain contains the constitutively expressed PDE gene *yhjH* leading to low internal levels of c-di-GMP, a condition important in swarming and swimming motility (Pesavento et al., 2008; Chua et al., 2013).

We found that low intracellular c-di-GMP content induced expression of the QS systems, specifically the *rhl* and *pqs* systems, which led to increased production of several virulence factors, such as rhamnolipids and pyocyanin. This was correlated to increased killing of macrophages. We showed that the induction of *rhl* and *pqs* QS under conditions of low c-di-GMP levels, was mediated by PqsR, the transcriptional regulator of *pqs* QS.

Hence, our present study suggested that c-di-GMP-governed biofilm dispersal might liberate bacteria capable of producing virulence factors, so as to survive and protect themselves from the phagocytic immune cells in the host. Hence, as a strategy to prevent the dissemination of biofilm infections, the use of QS inhibitors (Hentzer et al., 2003) can potentially reduce the production of QS-related virulence factors.

MATERIALS AND METHODS

Strains, Plasmids, and Growth Conditions

The bacterial strains and plasmids used in this study are listed in Table S1. *Escherichia coli* DH5 α strain was used for standard DNA manipulations (Bertani, 1951). LB medium was used to cultivate *E. coli* strains. Batch cultivation of *Pseudomonas aeruginosa* strains was carried out at 37°C in ABT minimal medium (Clark, 1968) with 5 g L⁻¹ glucose (ABTG) or 2 g L⁻¹ glucose and 2 g L⁻¹ casamino acids (ABTGC). To maintain plasmids in *E. coli*, 2 ml LB was supplemented with 100 μ g ampicillin (Ap) mL⁻¹, 15 μ g mL⁻¹ gentamicin (Gm), 15 μ g mL⁻¹ tetracycline (Tc), or 8 μ g mL⁻¹ chloramphenicol (Cm). In *P. aeruginosa*, 30 μ g mL⁻¹ Gm, 50 μ g mL⁻¹ Tc, and 200 μ g carbenicillin mL⁻¹ (Cb) were used for marker selection.

Quantification of c-di-GMP

Bacterial cells in 5 ml ABTGC were harvested and pelleted by centrifugation at 13,000 g for 3 min. The supernatant was removed and the cell pellet was immediately snap-frozen in liquid nitrogen. The cell pellet was re-suspended in 1 ml of acetonitrile/methanol/water (40:40:20) mixture. An aliquot of cells (10 μ l) was used for protein quantification. The cells were then lysed with a probe tip ultrasonicator (Amplitude 30%; 5 s ON, 5 s OFF) for 1 min in ice slurry. The cell debris was removed by centrifugation at 13000 g for 3 min. The liquid phase was then evaporated by using the vacuum concentrator, leaving behind the nucleotide precipitate. The samples were then re-suspended in 100 μ l ddH₂O and centrifuged at 10,000 g, 4°C for 10 min. The solutions were transferred to glass vials and injected through liquid chromatography-mass spectrometry (LCMS).

For the LC portion, the samples in the glass vials were run through the BEH C18 (1.7 μ m; 2.1 \times 50 mm) column with injection volume of 5 μ l at 0.3 ml min⁻¹ for a total runtime of 6 min, with the mobile phase A as 10 mM ammonium formate in water + 0.1% formic acid and mobile phase B as methanol + 0.1% formic acid. For the MS portion, the samples were then analyzed by Xevo TQ-S, Waters mass spectrometer, under the

ESI positive ion mode (capillary voltage: 3.8 kV, desolvation temperature: 400°C). The cyclic di-GMP compound was detected by monitoring ion transition of 691.2 m/z to 152.0 m/z at collision energy 36 eV.

For protein quantification, the cell aliquot was treated in 1 ml of 5 M NaOH at 95°C for 5 min. After cooling the samples for 15 mins, the proteins were processed with the Qubit® protein assay kit (NanoOrange dye) and quantified by the Qubit® 2.0 Fluorometer (Invitrogen). The concentration of c-di-GMP was then normalized with protein quantity. Experiments were performed in triplicate, and results were shown as the mean ± s.d.

Quantification of PDE Activity

Bacterial cells in 5 ml ABTGC were harvested and pelleted by centrifugation at 13,000 g for 3 min. The supernatant was removed and the cell pellet was resuspended in 5 ml 0.9% NaCl. The cells were lysed with a probe tip ultrasonicator (Amplitude 30%; 5 s ON, 5 s OFF) for 3 min in ice slurry to obtain a crude extract. As previously described (Kuchma et al., 2007), the crude extracts were incubated with 5 ml of 5 mM bis(p-nitrophenyl) phosphate (bis-pNPP) in buffer (5 mM MgCl₂, 50 mM Tris-HCl [pH 9.3], 50 mM NaCl). The release of p-nitrophenol was quantified by using a microplate reader (Tecan Infinite Series 2000) at OD410 every 15 min for 16 h.

As described in the previous section, protein concentration was determined by the Qubit® 2.0 Fluorometer (Invitrogen). The PDE activity was then normalized with protein quantity. Experiments were performed in triplicate, and results were shown as the mean ± s.d.

RNA Preparation

PAO1, PAO1ΔwspF and PAO1/*p_{lac}-yhjH* were grown in 1 ml ABTGC in each well (triplicates) within a 24-well microplate (Nunc) for 7 h till late logarithmic phase in 37°C, 200 rpm shaking. Bacterial cells were first treated with RNA Protect (Qiagen, Netherlands) and then treated with lysozyme. Total RNA was extracted using RNeasy Mini Kit (Qiagen, Netherlands). On-column DNase digestion with the RNase-free DNase Set (Qiagen) was used to remove DNA. The DNA contamination levels were assessed by using the Qubit® dsDNA High Sensitivity (HS) assay (PicoGreen dye) and the Qubit® 2.0 Fluorometer (Invitrogen). The integrity of total RNA was assessed by using the Bioanalyser RNA analysis kit (Agilent Technologies) and the Agilent 2100 Bioanalyzer (Agilent Technologies). The Ribo-Zero™ Magnetic Kit (Bacteria) (Epicentre) was used to deplete 16S, 23S, and 5S rRNAs from the samples.

RNA Sequencing and Data Analysis

Gene expression analysis of 2 biological replicates was conducted by RNA-Seq technology (Illumina). The RNA was fragmented to 200–300 bp fragments using divalent cations under elevated temperature.

First and second strand cDNA were then synthesized and treated by end repair and adapter ligation. After the 12-cycle PCR enrichment, the quality of the libraries was assessed using the Agilent 2100 Bioanalyzer (Agilent Technologies). The libraries

were sequenced using the Illumina HiSeq2000 platform with paired-end protocol and read lengths of 100 nt.

The sequence reads were assembled and analyzed by “RNA-Seq and expression analysis” application of CLC genomics Workbench 6.0 (CLC Bio, Aarhus, Denmark). The PAO1 genome (<http://www.ncbi.nlm.nih.gov/nucore/110645304>) was utilized as the reference genome. The following criteria were used to filter the unique sequence reads: maximum number of hits for a read of 1, minimum length fraction of 0.9, minimum similarity fraction of 0.8, and maximum number of two mismatches. Genes were annotated with Pseudomonas Genome Database (Winsor et al., 2011). The mapping results of RNA-Seq raw data from CLC genomics Workbench 6.0 were subjected to DESeq2 package for statistical analysis (Anders and Huber, 2010) by reading them into R/Bioconductor (Gentleman et al., 2004). The transcript counts were normalized to the effective library size. Hierarchical clustering analysis was performed and a heatmap was drawn for the 1000 most highly expressed genes of PAO1, PAO1ΔwspF, and PAO1/*p_{lac}-yhjH* using heatmap.2 package of R/Bioconductor (Gentleman et al., 2004). Furthermore, the normalized counts were stabilized according their variance as outlined in the DESeq2 package tutorial and a principle component analysis (PCA) plot was generated. The differentially expressed genes among PAO1, PAO1ΔwspF, and PAO1/*p_{lac}-yhjH* were identified by performing a negative binomial test using the DESeq2 package of R/Bioconductor. Transcripts were stringently determined as differentially expressed when having a fold change larger than 5 and an adjusted *p*-value smaller than 0.05.

Accession number for the RNA-seq is PRJNA381683.

qRT-PCR Analysis

Total RNA from cells grown in 2 ml ABTGC was extracted using RNeasy Mini Kit (Qiagen) with on-column DNase digestion. The concentration and purity of the extracted RNA were measured by NanoDrop 2000 spectrophotometer (Thermo Scientific), while the integrity of RNA was analyzed by Agilent 2200 TapeStation System (Agilent Technologies). The elimination of contaminating DNA was confirmed via real time PCR amplification of the *rpoD* gene with total RNA as template.

First-strand cDNA was first synthesized from total RNA with the SuperScript® III First-Strand Synthesis SuperMix kit (Invitrogen). The cDNA was used as template for qRT-PCR with a kit of SYBR® Select Master Mix (Applied Biosystems, Life Technologies) on the StepOnePlus Real-Time PCR System (Applied Biosystems, Life Technologies). The gene *rpoD* was used as endogenous control. To verify specific single-product amplification, melting curves were analyzed.

Quantification of BHL by Using *ΔlasIΔrhII/p_{rhlA}-gfp* Reporter Fusion

Supernatants (2 ml) from *P. aeruginosa* strains grown in ABTGC in 37°C overnight were filtered through 0.2-μm filters, and the filtrates were collected. Overnight culture of the reporter strain *ΔlasIΔrhII/p_{rhlA}-gfp* was adjusted to OD₆₀₀ = 0.2 using ABTGC medium. 100 μl of filtrate was added to 100 μl of *ΔlasIΔrhII/p_{rhlA}-gfp* in a 96-well plate (Nunc, Denmark). Because *ΔlasIΔrhII* does not produce BHL, *p_{rhlA}-gfp* was

induced by the addition of serial diluted filtrates containing BHL. GFP fluorescence from *p_{rhIA}-gfp* expression (expressed in relative fluorescence units, RFU) was measured for each well using a microplate reader (Tecan Infinite 2000) and was normalized to the OD₆₀₀ of each well. Experiments were performed in triplicate, and results are shown as the mean \pm s.d.

Quantification of PQS by Using $\Delta pqsA/p_{pqsA}$ -gfp Reporter Fusion

Supernatants (2 ml) from *P. aeruginosa* strains grown in ABTGC in 37°C overnight filtered through 0.2- μ m filters and the filtrates were collected. Overnight culture of the reporter strain $\Delta pqsA/p_{pqsA}$ -gfp was adjusted to OD₆₀₀ = 0.2 using ABTGC medium. 100 μ l of filtrate was added to 100 μ l of $\Delta pqsA/p_{pqsA}$ -gfp in a 96-well plate (Nunc, Denmark). Because $\Delta pqsA$ does not produce PQS, *p_{pqsA}-gfp* was induced by the addition of serial diluted filtrates containing PQS. GFP fluorescence from *p_{pqsA}-gfp* expression (expressed in relative fluorescence units, RFU) was measured for each well using a microplate reader (Tecan Infinite 2000) and was normalized to the OD₆₀₀ of each well. Experiments were performed in triplicate, and results are shown as the mean \pm s.d.

Quantification of Rhamnolipids by Orcinol Assay

Relative amounts of rhamnolipids produced by *P. aeruginosa* strains were quantified as previously described (Wittgens et al., 2011; Fong et al., 2016). The supernatant of strain grown in 2 ml ABTGC in 37°C overnight was filtered with 0.2- μ m filter and treated with equal volumes of ethyl acetate. Samples were then mixed with vortexing for 30 s, with a phase separation by putting samples briefly in centrifuge for 30 s at 5,000 g. The upper organic phase containing rhamnolipids were transferred to new tube. The organic solvent was then removed by evaporation with a vacuum concentrator. The residue containing rhamnolipids was then re-suspended in 100 μ l ddH₂O. 100 μ l of sample was mixed with 100 μ l of 1.6% orcinol in ddH₂O and 800 μ l sulphuric acid (60% v/v). The samples were then incubated at 80°C for 30 mins and mixed periodically. After cooling the samples to room temperature for 15 mins, OD₄₂₀ was measured by the microplate reader (Tecan Infinite 2000). Experiments were performed in triplicates and results were shown as mean \pm standard deviation.

Quantification of Pyocyanin

Relative levels of pyocyanin produced by *P. aeruginosa* strains were quantified as previously described (Frank and Demoss, 1959; Fong et al., 2016). The supernatant of strain grown in 2 ml ABTGC in 37°C overnight was filtered with 0.2- μ m filter and treated with equal volumes of chloroform. Samples were then mixed with vortexing for 30 s, with a phase separation by putting samples in centrifuge for 30 s at 5,000 g. The lower organic phase containing pyocyanin was transferred to new tube. 200 μ l of 0.1 M hydrochloric acid (HCl) was then added to the chloroform phase and mixed with vortexing for 30 s, with a phase separation by putting samples briefly in centrifuge. The pink coloration which subsequently formed by acidification of pyocyanin, in

the HCl phase at the top layer was then transferred to a 96-well microplate (Nunc, Denmark) and OD₅₀₀ was measured by the microplate reader (Tecan Infinite 2000). Experiments were performed in triplicates and results were shown as mean \pm standard deviation.

Macrophages

The murine macrophage cell line RAW264.7 (ATCC No. TIB-71) was grown in 15 ml Dulbecco's Modified Eagle's Medium (DMEM) (Life Technologies), supplemented with 10% fetal bovine serum (FBS) (Gibco). Cells were incubated in 75 cm² cell culture flasks (Nunc, Denmark) at a density of 5.0×10^6 cells ml⁻¹ for 72 h, at 37°C, 5% CO₂ and 90% humidity.

The cells were checked against *Mycoplasma* contamination by using the PCR *Mycoplasma* detection kit (Abmgood, USA) before experiments.

Macrophage Cytotoxicity Assay

To test the ability of cells to kill macrophages, cytotoxicity of macrophages was determined by monitoring cell integrity in 1 ml DMEM + 10% FBS + 20 μ M propidium iodide (PI), as previously described (Chua et al., 2016b). Cells that stained by PI under the epifluorescent microscopy (Zeiss) with 20 \times objective were observed as dead. The ratio of dead cells to live cells, enumerated from five images (each image contained approximately 200 macrophages), was then calculated. Experiments were performed in triplicates and results were shown as mean \pm standard deviation.

RESULTS

Comparing the c-di-GMP Content of PAO1 Cells with High and Low c-di-GMP Levels

We used RNA-sequencing to compare the transcriptomes of *P. aeruginosa* PAO1 cells "locked" in conditions of either high or low intracellular c-di-GMP contents. The late log phase *P. aeruginosa* $\Delta wspF$ mutant cells possessed a high intracellular c-di-GMP content due to the constitutively expressed WspR DGC protein (D'argenio et al., 2002; Rybtke et al., 2012; Chua et al., 2016a; **Figure 1A**), contributing to the overproduction of exopolysaccharides and low motility in $\Delta wspF$ cells. Hence, we used $\Delta wspF$ cells to mimic the biofilm stage. On the other hand, the late log phase *P. aeruginosa* *p_{lac}-yhjH* mutant cells contained reduced c-di-GMP content due to the constitutively expressed YhjH PDE protein (Gjermansen et al., 2010; Chua et al., 2013; **Figure 1A**), thus mimicking cells freshly dispersed from the biofilms. We also corroborated our findings by detecting increased enzymatic PDE activity in the *p_{lac}-yhjH* harboring cells, as compared to wild type and $\Delta wspF$ mutant cells (**Figure 1B**).

Comparing the Transcriptomes of PAO1 Cells with High and Low c-di-GMP Levels

The $\Delta wspF$ and PAO1/*p_{lac}-yhjH* mutants demonstrated distinct gene expression profiles according to the heat map diagram and PCA analysis (**Figures 2A,B**). 431 genes were up-regulated and 595 genes were down-regulated in the *p_{lac}-yhjH* mutant as compared to the $\Delta wspF$ mutant (Data Sheet 1), including

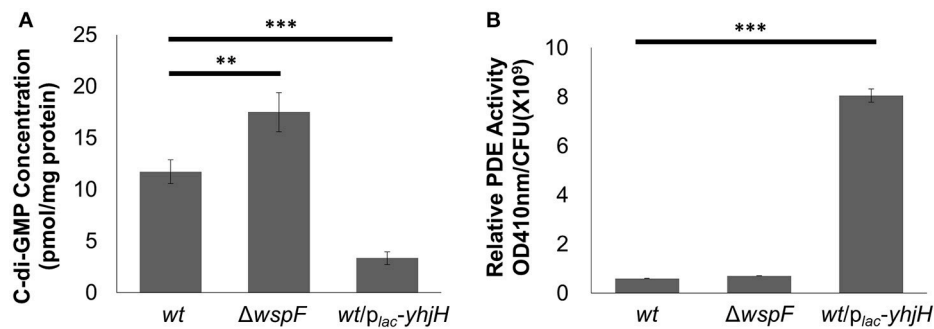


FIGURE 1 | Higher PDE activity in *P. aeruginosa*/ p_{lac} -*yhjH* correlated to lower c-di-GMP levels. **(A)** C-di-GMP quantification by LCMS in *P. aeruginosa* wt, $\Delta wspF$ and *P. aeruginosa*/ p_{lac} -*yhjH*. **(B)** PDE activity in *P. aeruginosa* wt, $\Delta wspF$ and *P. aeruginosa*/ p_{lac} -*yhjH*. Means and s.d. from triplicate experiments are shown. ** $P < 0.01$, *** $P < 0.001$, Student's *t*-test.

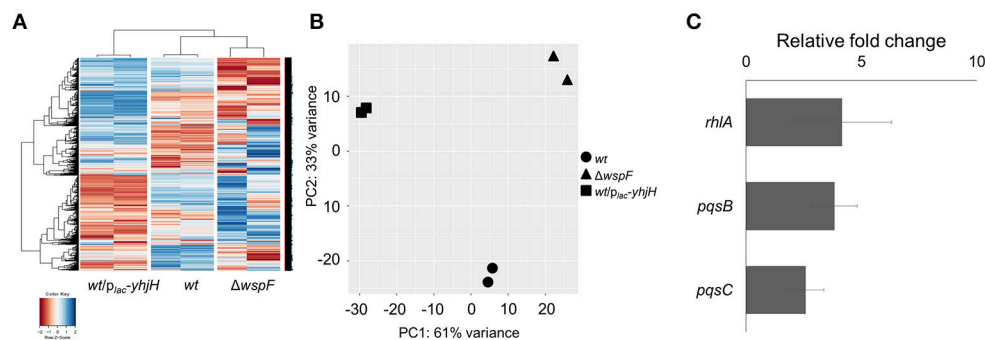


FIGURE 2 | Comparison of gene expression by the *P. aeruginosa* wild-type, high intracellular c-di-GMP containing $\Delta wspF$ mutant and low intracellular c-di-GMP containing *P. aeruginosa*/ p_{lac} -*yhjH* mutant. **(A)** Heat map and **(B)** PCA plot comparison of wild-type, $\Delta wspF$ and *P. aeruginosa*/ p_{lac} -*yhjH*. **(C)** qRT-PCR analysis of *rhlA*, *pqsB* and *pqsC* expression in *P. aeruginosa*/ p_{lac} -*yhjH* culture relative to $\Delta wspF$ strain. Means and s.d. from triplicate experiments are shown.

genes regulated by QS (in particular regulated by the *rhl* and *pqs* encoded systems). Specifically, *rhlR*, *rhlA* and *rhlB* were highly induced in the p_{lac} -*yhjH* strain compared to the $\Delta wspF$ mutant (Data Sheet 1). This finding was validated by qRT-PCR analysis (Figure 2C) and the *rhl* QS reporter fusion p_{rhlA} -*gfp* (Yang et al., 2009; Figure 3A). Accordingly, the p_{lac} -*yhjH* strain was found to produce more rhamnolipids (Figure 3B), PQS (Figure 3C) and pyocyanin (Figure 3D) than the $\Delta wspF$ mutant, and was thus more cytotoxic to murine macrophages than the $\Delta wspF$ mutant (Figure 3E). This showed that the reduction of c-di-GMP levels lead to induction of the QS systems, which in turn stimulate production of a subset of QS-controlled virulence factors. Conversely, the $\Delta wspF$ mutant with the “mimicked” biofilm phenotype had higher expression of biofilm-related genes important for exopolysaccharide production (*pelB*, *pelC*, *pelD* and *PelF*) and siderophore production (*pvdD*, *pvdJ*, *pvdL*, *pvdO*), as compared to the PAO1/ p_{lac} -*yhjH* mutant.

PqsR Induces the *pqs* and *rhl* QS

We also noticed that genes from the *pqs* operon, such as *pqsA*, *pqsB*, *pqsC*, and *pqsH* were highly induced in cells with low intracellular c-di-GMP content (Data Sheet 1). The qRT-PCR analysis confirmed these findings (Figure 2C). Since

the *pqs* operon is controlled by PqsR, we next hypothesized that PqsR could be crucial to the induction of *pqs* QS system under conditions of low c-di-GMP levels. Since the *rhl* encoded QS system can be induced by the *pqs* QS system (McKnight et al., 2000), it was also possible that activation of the *pqs* QS system boosted rhamnolipid production. Furthermore, a $\Delta pqsR/p_{lac}$ -*yhjH* mutant produced lesser rhamnolipids than the p_{lac} -*yhjH* mutant (Figure 4A). Accordingly, we showed that a PqsR deficient $\Delta pqsR/p_{lac}$ -*yhjH* mutant expressed only low levels of pyocyanin, BHL and PQS compared with the p_{lac} -*yhjH* mutant (Figures 4B–D). This correlated well with lower cytotoxicity to macrophages by $\Delta pqsR$ (Figure 4E). Hence, PqsR appeared to be a key regulator for induction of QS systems by low intracellular c-di-GMP content.

DISCUSSION

While most studies focused on the effects of high c-di-GMP levels on biofilm formation, there is a paucity of research on the physiology of cells undergoing conditions of low c-di-GMP signaling. Our previous study had shown that cells freshly dispersed from biofilms contained lower c-di-GMP levels

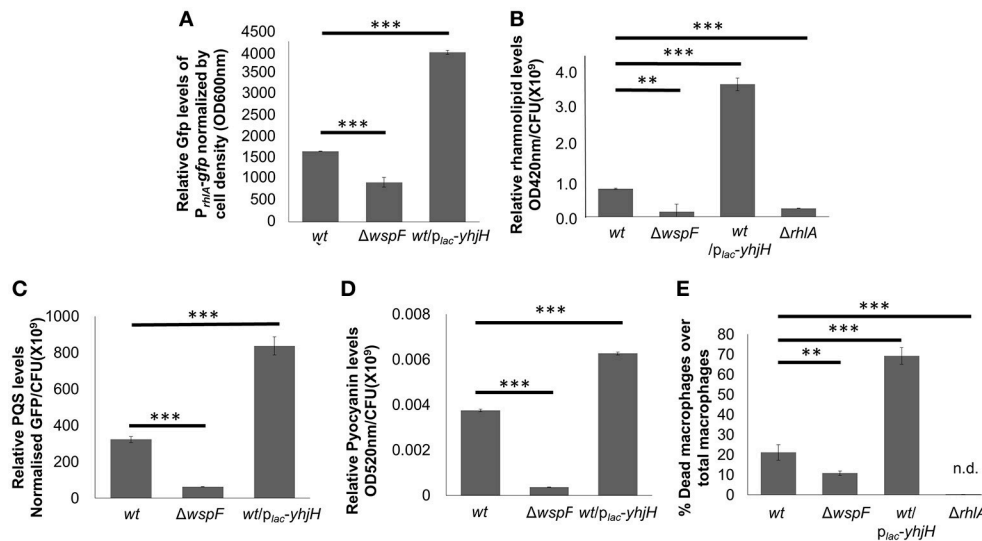


FIGURE 3 | Induction of QS-controlled virulence factors in low intracellular c-di-GMP containing *P. aeruginosa*/*p_{lac}-yhjH* mutant. Relative quantification of *P_{rhlA}*-ASV (A), rhamnolipids (B), PQS (C) and pyocyanin (D) in PAO1, $\Delta wspF$ mutant and *P. aeruginosa*/*p_{lac}-yhjH* mutant. (E) Cytotoxicity of *P. aeruginosa*/*p_{lac}-yhjH* and $\Delta wspF$ to macrophages. Means and s.d. from triplicate experiments are shown. ***P* < 0.01, ****P* < 0.001, One-way ANOVA.

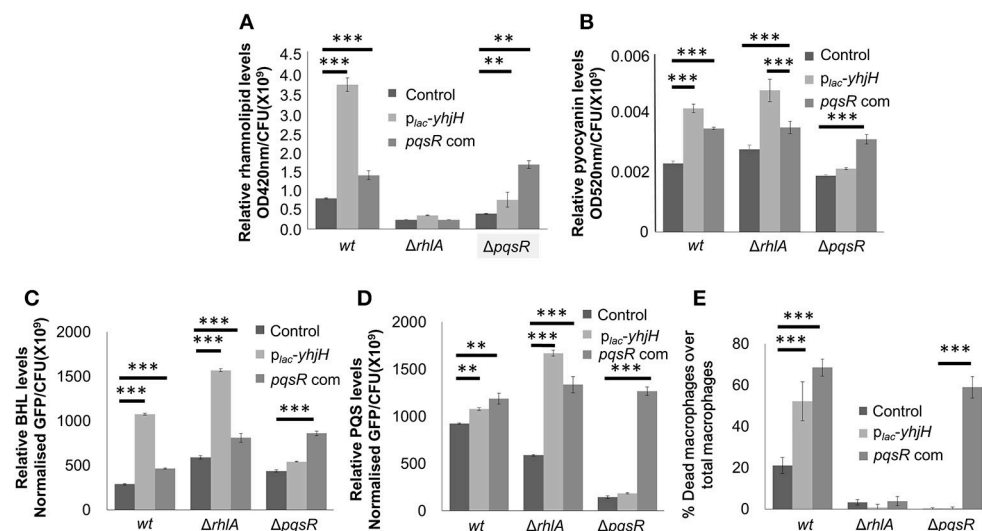


FIGURE 4 | PqsR links c-di-GMP signaling to *rhl* quorum sensing. Rhamnolipid (A), pyocyanin (B), BHL (C) and PQS (D) quantification of *P. aeruginosa* wt and $\Delta pqsR$ cultures. (E) Cytotoxicity of *P. aeruginosa*/*p_{lac}-yhjH* and $\Delta pqsR$ against macrophages. Means and s.d. from triplicate experiments are shown. ***P* < 0.01, ****P* < 0.001, One-way ANOVA.

than planktonic cells and biofilm cells (Chua et al., 2013), implying that dispersed cells possess a different physiology from biofilm and planktonic cells. This raised the question of how differing c-di-GMP levels impact the physiology in *P. aeruginosa*. As biofilm cells and dispersed cells had high physical and physiological heterogeneity (Stewart and Franklin, 2008), we used the $\Delta wspF$ and PAO1/*p_{lac}-yhjH* mutants to imitate biofilm and dispersed cells respectively, and cultivated them as planktonic cultures which were easy to manipulate in controlled conditions.

In this work, we compared the transcriptomics of cells with high and low c-di-GMP levels. Other than biofilm dispersal, we had shown using transcriptomics that low c-di-GMP levels could lead to the induction of the *pqs* and *rhl* QS, with PqsR acting as mediator to activate both QS systems. Although we do not show that conditions of low c-di-GMP mediate increased PqsR, a previous study had shown that RsmA from the c-di-GMP-mediated Gac/Rsm pathway, was important in *pqs* and *rhl* QS (Burrowes et al., 2006). Hence, the result of activating both QS systems was the increased production of pyocyanin

and rhamnolipids, which were correlated to higher virulence to immune cells. Interestingly, it was previously observed that rhamnolipids acted as surfactant to facilitate biofilm cells to disperse from biofilms (Bhattacharjee et al., 2016).

Several research groups, such as ours (Chua et al., 2015; Yu et al., 2015) are currently investigating the possibility of exploiting the lowering of the c-di-GMP content in bacteria and dispersal as a biofilm control strategy. Our study had several implications for clinical and environmental applications of this biofilm dispersal strategy. Firstly, liberated bacterial cells could attain a unique physiological state if the c-di-GMP content is maintained at a lower level than planktonic cells and biofilm cells. This state could be reached after long-term growth of dispersed biofilm cells in the presence of agents that cause biofilm dispersal, thus warranting further studies on the biofilm-dispersed cells. Secondly, it appeared that a constitutively low c-di-GMP content renders the bacterial cells highly virulent, which might be essential for dispersed cells to survive the encounter with immune cells and cause development of sepsis.

Hence, it is important to evaluate the potential virulence outcome which applying c-di-GMP mediated biofilm dispersal during the eradication of biofilms, especially in infections. The use of QS inhibitors (Hentzer et al., 2003) can effectively negate the induction of QS pathways and production of virulence factors, to be used concurrently with c-di-GMP-mediated biofilm dispersal.

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AUTHOR CONTRIBUTIONS

SL and LY designed methods and experiments, analyzed the data, interpreted the results and wrote the paper. YL designed RNA-seq experiments, discussed analyses, interpretation, and presentation. YYL and HJ performed experiments for qRT-PCR work for analysis and interpretation. GK performed data processing for RNA-seq. SL, TT, LY and MG defined the research theme and discussed project outline. All authors have contributed to, seen and approved the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fcimb.2017.00451/full#supplementary-material>

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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